

# VeA and MvIA repression of the cryptic orsellinic acid gene cluster in *Aspergillus nidulans* involves histone 3 acetylation

Jin Woo Bok,<sup>1</sup> Alexandra A. Soukup,<sup>2</sup>  
Elizabeth Chadwick,<sup>1</sup> Yi-Ming Chiang,<sup>4,5</sup>  
Clay C. C. Wang<sup>5,6</sup> and Nancy P. Keller<sup>1,3\*</sup>

Departments of <sup>1</sup>Medical Microbiology and Immunology,  
<sup>2</sup>Genetics and <sup>3</sup>Bacteriology, University of Wisconsin,  
Madison, WI, USA.

<sup>4</sup>Graduate Institute of Pharmaceutical Science, Chia  
Nan University of Pharmacy and Science, Tainan  
71710, Taiwan.

Departments of <sup>5</sup>Pharmacology and Pharmaceutical  
Sciences and <sup>6</sup>Chemistry, University of Southern  
California, 1985 Zonal Avenue, Los Angeles, CA 90033,  
USA.

## Summary

A perplexing aspect of fungal secondary metabolite gene clusters is that most clusters remain 'silent' under common laboratory growth conditions where activation is obtained through gene manipulation or encounters with environmental signals. Few proteins have been found involved in repression of silent clusters. Through multicopy suppressor mutagenesis, we have identified a novel cluster suppressor in *Aspergillus nidulans*, MvIA (modulator of *veA* loss). Genetic assessment of MvIA mutants revealed the role of both itself and VeA (but not the VeA partner LaeA) in the suppression of the cryptic *ors* gene cluster producing orsellinic acid and its F9775 derivatives. Loss of *veA* upregulates F9775A and F9775B production and this increase is reduced 4–5-fold when an overexpression *mvIA* (*OE:mvIA*) allele is introduced into the  $\Delta veA$  background. Previous studies have implicated a positive role for GcnE (H3K9 acetyltransferase of the SAGA/ADA complex) in *ors* cluster expression and here we find expression of *gcnE* is upregulated in  $\Delta veA$  and suppressed by *OE:mvIA* in the  $\Delta veA$  background. H3K9 acetylation levels of *ors* cluster genes correlated with *gcnE* expression and F9775 production in  $\Delta veA$  and *OE:mvIA* $\Delta veA$  strains. Finally, deletion of *gcnE* in the  $\Delta veA$  background abolishes *ors* cluster activation and F9775 production. Together, this work supports

a role for VeA and MvIA in modifying SAGA/ADA complex activity.

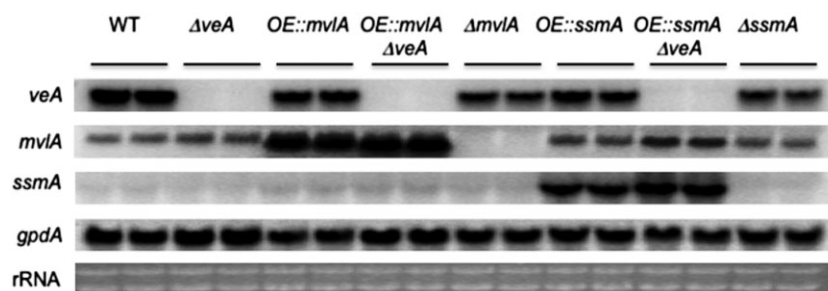
## Introduction

Secondary metabolism is a process by which plants, fungi, and bacteria naturally produce chemicals that are used in niche specialization (Demain and Fang, 2000). As opposed to primary metabolites, secondary metabolites are not necessary for laboratory growth conditions but appear to afford defensive/protective properties to the producing organism (Rohlf and Churchill, 2011; Yin *et al.*, 2012). Secondary metabolites are of great interest to humankind because they can be both beneficial (e.g. pharmaceuticals) and harmful (e.g. mycotoxins) to human and animal health. This interest has led to many studies aimed at understanding the genetic regulation of these bioactive metabolites.

Secondary metabolism in filamentous fungi is globally regulated by a conserved heterotrimeric protein complex called the Velvet Complex (Bayram *et al.*, 2008; Amaike and Keller, 2009; Hoff *et al.*, 2010; Wiemann *et al.*, 2010; López-Berges *et al.*, 2012; Wu *et al.*, 2012; Yang *et al.*, 2013). This complex is composed of the LaeA, VeA, and VelB proteins. Deletion of these genes decreases secondary metabolite production but the phenotypes of the deletants are not equivalent. Loss of *laeA* and *veA* – the two genes most studied – have a major impact on secondary metabolism in *Aspergillus nidulans* (Bayram *et al.*, 2008). *laeA* encodes a putative nuclear methyltransferase which positively regulates metabolic gene clusters and controls expression of metabolites such as sterigmatocystin, aflatoxin, penicillin, lovastatin, terraquinone, and mycelial pigments, with deletion strains reduced and overexpression strains increased in metabolite production (Bok and Keller, 2004). Loss of *veA*, also called the 'velvet' gene and encoding a light regulated protein (Calvo *et al.*, 2002), results in decrease of many of the same metabolites but increase in mycelial pigmentation. In contrast to *laeA* overexpression, *veA* overexpression leads to decreased penicillin production (Spröte and Brakhage, 2007) and oppositely regulates certain penicillin cluster genes (Kato *et al.*, 2003).

These latter studies suggest that VeA can negatively regulate some secondary metabolite clusters through an

Accepted 8 July, 2013. \*For correspondence. E-mail npkeller@wisc.edu; Tel. (608) 262 9795; Fax (608) 262 8418.



**Fig. 1.** *veA*, *mvIA*, and *ssmA* gene expression in *A. nidulans* wild type (WT),  $\Delta veA$ , *OE::mvIA*, *OE::mvIA*  $\Delta veA$ ,  $\Delta mvIA$ , *OE::ssmA*, *OE::ssmA*  $\Delta veA$ , and  $\Delta ssmA$  strains (RDIT9.32, RJW112.2, RJW220.17, RJW221.5, REC1C, RJW216.15, RJW226.4, and RJW215.25 respectively) grown in liquid shaking GMM for 48 h at 37°C. RNA was extracted from independent biological duplicated samples. Ethidium bromide-stained rRNA and *gpdA* are indicated for loading.

unknown mechanism. Recent insight into positive regulation of secondary metabolite clusters by VeA and LaeA have arisen from genetic screens of deletants of either protein. Chemical mutagenesis of a  $\Delta veA$  strain resulted in the identification of *RtfA*, a RNA-pol II transcription elongation factor-like protein, which when deleted in a  $\Delta veA$  background resulted in the remediation of sterigmatocystin in a medium dependent manner (Ramamoorthy *et al.*, 2012). Loss of *rtfA* did not, however, rescue sterigmatocystin in a  $\Delta laeA$  strain. In a multicopy genetic suppressor screen of a  $\Delta laeA$  strain, overexpression of two genes was found to partially complement sterigmatocystin production. One gene encoded the bZIP transcription factor *RsmA* (restorer of secondary metabolism A) that specifically bound to the promoter of *affR*, the sterigmatocystin specific transcription factor (Yin *et al.*, 2012). Overexpression of *rsmA* also restored sterigmatocystin production in a  $\Delta veA$  background (Shaaban *et al.*, 2010). The other gene encoded *EsaA*, a histone 4 acetyltransferase involved in early activation of multiple secondary metabolite gene clusters (Soukup *et al.*, 2012a). This latter study supported earlier research correlating chromatin modifications with secondary metabolite cluster activation or repression (Roze *et al.*, 2007; Bok *et al.*, 2009; Reyes-Dominguez *et al.*, 2010; Strauss and Reyes-Dominguez, 2011).

Here, using the same multicopy suppressor mutagenesis strategy which identified *RsmA* and *EsaA* in the  $\Delta laeA$  background, we looked for genes impacting secondary metabolism in a  $\Delta veA$  genetic background with the thought that some of the genes would be identical to those found in the  $\Delta laeA$  screen. Although this was not the case, our screen revealed a novel protein termed *MvIA* (modulator of *veA* loss A). Analysis of both *mvIA* deletion and overexpression alleles in several genetic backgrounds allowed us to characterize both VeA and *MvIA* as negative regulators of the cryptic orsellinic acid (*ors*) secondary metabolite gene cluster. This regulation was associated with histone 3 modifications, particularly increased H3K9 acetylation by the histone 3 acetyltransferase SAGA/ADA complex member *GcnE*. Furthermore, *gcnE* deletion eliminated *ors* expression and product formation in the  $\Delta veA$  background. This is the first genetic screen, to our knowledge, to identify

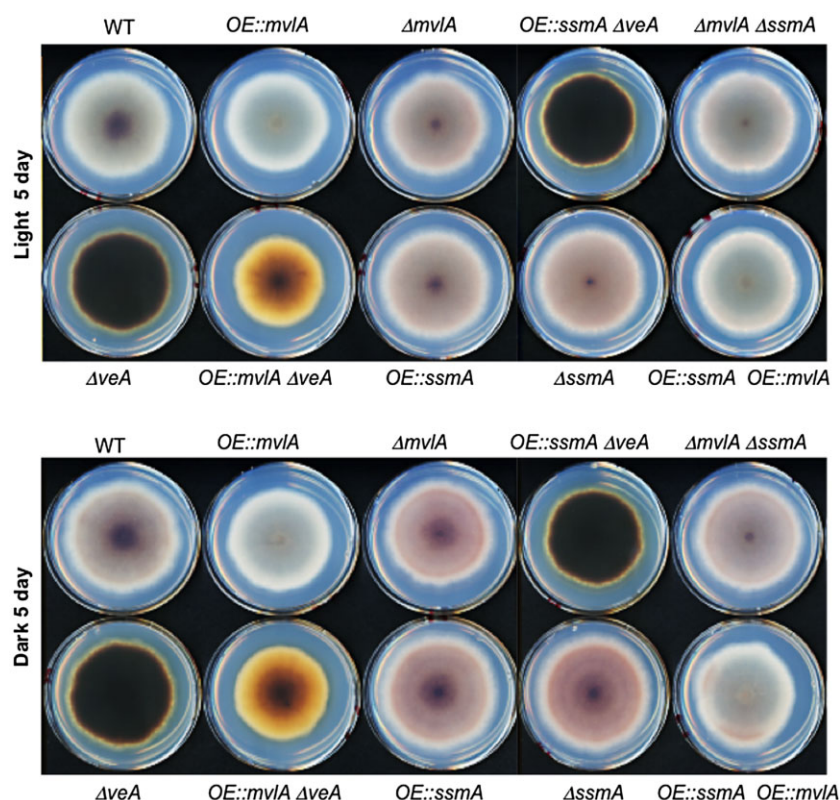
a novel negative regulator of a cryptic gene cluster as well as provide some insight into a mechanism for VeA negative regulation of a secondary metabolite cluster.

## Results

### Identification of *mvIA* as a moderator of *veA* loss

Transformation of RJW113.4 ( $\Delta veA$ , *pyrG89*) with the AMAI library generated 30 000 transformants, three of which partially remediated the  $\Delta veA$  phenotype as detected by pigment loss in mycelium as observed from the bottom side of Petri plates (Fig. S1). One of the plasmids rescued from one of the three transformants contained a single full-length open reading frame (ORF) of the gene AN8797, termed *mvIA* (modulator of *VeA* loss A), with a functional promoter and termination cassette, which maps to chromosome 3. *mvIA* encodes a 480 aa protein with considerable identity (43%) to *Urc4*, a protein recently characterized as important in uracil degradation in the yeast *Saccharomyces (Lachnaceae) kluyveri* (Andersen *et al.*, 2008; Björnberg *et al.*, 2010). Further BLAST searching identified another *A. nidulans* gene, AN12027, encoding a 429 aa protein with 44% identity to both *MvIA* and *Urc4*. All sequenced Aspergilli contain homologues of these two similar proteins.

To characterize *mvIA*, we overexpressed and deleted *mvIA* in wild type and overexpressed *mvIA* in a  $\Delta veA$  background (Fig. S2A). With the thought that AN12027 could possibly be playing a similar role as *mvIA*, we also deleted and overexpressed this gene, hence called *ssmA* (similar sequence to *MvIA*), in wild type and also overexpressed *ssmA* in a  $\Delta veA$  background (Fig. S2B). Northern data confirmed high expression of *mvIA* and *ssmA* by their respective overexpression alleles (Fig. 1). Similar to the original AMAI mutant, overexpression of *mvIA* in the  $\Delta veA$  background decreased pigmentation but overexpression of *ssmA* had no effect on pigmentation (Fig. 2) leading us to conclude the two proteins did not fulfill the same roles in the fungus. Localization of *MvIA* was determined by microscopy of a *mvIA::eGFP* strain. The protein was evenly dispersed throughout the cytoplasm (Fig. S3).



**Fig. 2.** Mycelial pigmentation patterns of *A. nidulans* WT,  $\Delta veA$ ,  $OE::mvIA$ ,  $OE::mvIA\Delta veA$ ,  $\Delta mvIA$ ,  $OE::ssmA$ ,  $OE::ssmA\Delta veA$ ,  $\Delta ssmA$ ,  $\Delta mvIA\Delta ssmA$ , and  $OE::mvIAOE::ssmA$  strains (RDIT9.32, RJW112.2, RJW220.17, RJW221.5, REC1C, RJW216.15, RJW226.4, RJW215.25, RJW229.2, and RJW228.3 respectively) strains grown on solid GMM at light and dark for 5 days at 37°C.

#### Both *mvIA* and *veA* negatively regulate the cryptic *ors* gene cluster

Loss of either *veA* or *laeA* results in decreased levels of multiple secondary metabolites including the well-known metabolites sterigmatocystin and penicillin (Kato *et al.*, 2003; Bok and Keller, 2004). Neither of these metabolites was greatly impacted by either *mvIA* overexpression or *mvIA* loss in wild type,  $\Delta veA$  or  $\Delta laeA$  backgrounds as determined by TLC and bioassay (Fig. S4 and data not shown). As  $\Delta veA$  and  $\Delta laeA$  phenotypes themselves are not equivalent, with  $\Delta veA$  but not  $\Delta laeA$  exhibiting a dark pigmentation, there was no change of pigmentation in  $OE::mvIA\Delta laeA$  as compared to  $\Delta laeA$  alone (data not shown).

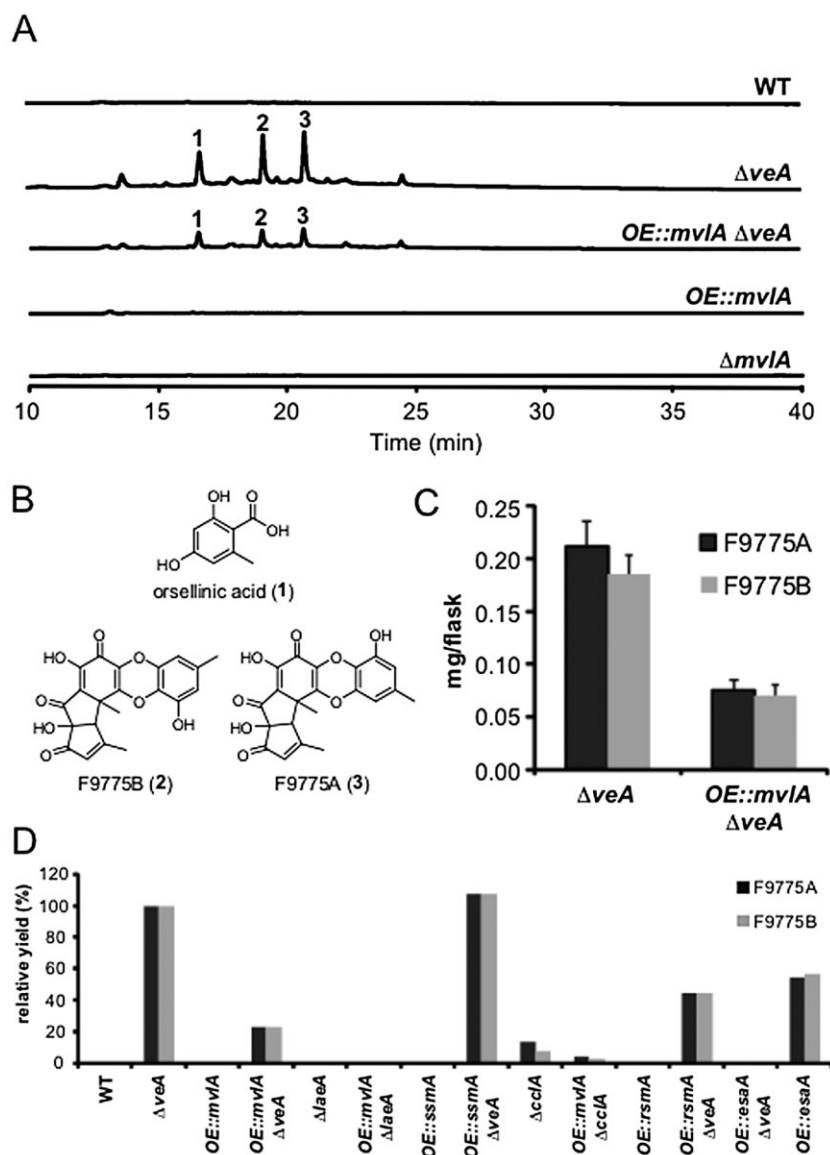
Postulating that natural products were responsible for the pigmentation of the  $\Delta veA$  strain, we next examined extracts from WT,  $\Delta veA$ ,  $OE::mvIA\Delta veA$ ,  $OE::mvIA$ , and  $\Delta mvIA$  by LC/MS for impact on other secondary metabolites (Fig. 3). These data clearly showed a dramatic increase in the production of the polyketide orsellinic acid and its derivatives, the cathepsin K inhibitors F9775A and F9775B, in the  $\Delta veA$  strain. This increase was dampened in the  $OE::mvIA\Delta veA$  strain. The increase in orsellinic acid and F9775 production in  $\Delta veA$  was accompanied by an increase in gene expression of the *ors* secondary metabolite gene cluster (*orsA-E*) responsible for the production of orsellinic acid and F9775 (Fig. 4). Paralleling the

chemistry, *ors* cluster gene expression was delayed in the  $OE::mvIA\Delta veA$  strain. The dark pigmentation of the  $\Delta veA$  strain thus appeared to be due to in part to high production of *ors* cluster derived products. This was confirmed by deleting *orsA* encoding the polyketide synthase required for production of orsellinic acid and F9775 (Sanchez *et al.*, 2010) in the  $\Delta veA$  background. The double  $\Delta veA\Delta orsA$  strain yielded a much lighter mycelial pigmentation phenotype similar in appearance to the  $OE::mvIA\Delta veA$  strain (Fig. S5A). Also *veA* loss did not increase expression of other genes associated with fungal pigmentation, further supporting the contribution of *ors* products to the dark coloration of  $\Delta veA$  (Fig. S6).

#### *MvIA* and *VeA* connection with histone 3 modification and *GcnE* activity

The *ors* gene cluster encoding for F9775 was one of the first cryptic gene clusters to be characterized in *A. nidulans* (Bok *et al.*, 2009; Schroeckh *et al.*, 2009). In these previous studies, a role for chromatin remodelling enzymes had been implicated in *ors* expression. Loss of CclA, a member of the Compass Complex required for histone 3 lysine 4 (H3K4) methylation, had been found to result in derepression of several secondary metabolite clusters including the *ors* cluster, the latter leading to increased F9775 production (Bok *et al.*, 2009). This study also established cross-





**Fig. 3.** Overexpression of *mvIA* suppresses *ors* gene cluster activated in  $\Delta veA$  strain.

A. Total scan HPLC profile of secondary metabolites extracted from the culture medium of *A. nidulans* WT,  $\Delta veA$ , OE::mvIA, OE::mvIA $\Delta veA$ , and  $\Delta mvIA$  (RDIT9.32, RJW112.2, RJW220.17, RJW221.5, and REC1C respectively) strains.

B. Metabolites identified from  $\Delta veA$  strain.

C. The yield of F9775A and B from  $\Delta veA$  and OE::mvIA  $\Delta veA$  strains in 48 h GMM liquid culture.

D. Relative yield of F9775A and B from extracts obtained from 48 h liquid culture media of the listed strains as compared to  $\Delta veA$ .

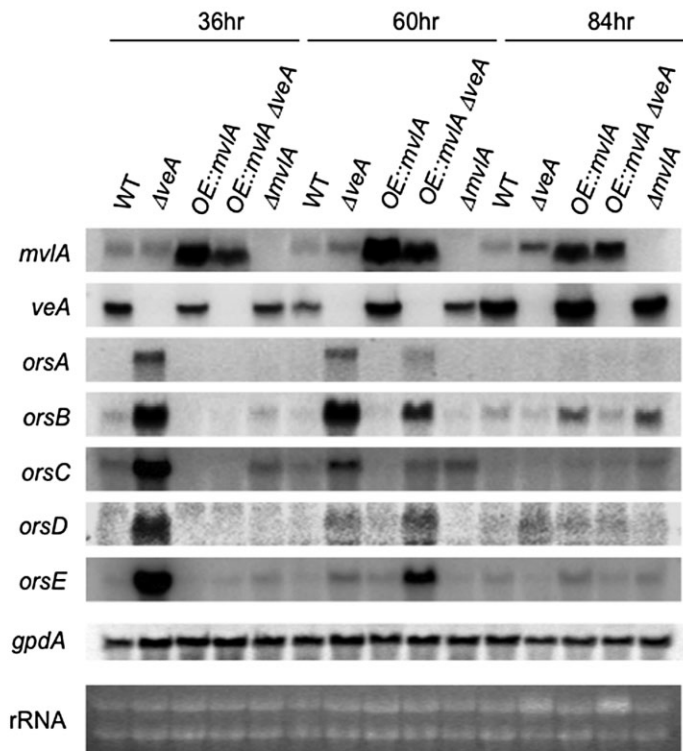
talk with H3K4 and H3K9 modifications as methylation of both lysine residues was decreased in the derepressed clusters. *Streptomyces rapamycinicus* induction of the *ors* cluster was found to be dependent on the histone 3 acetyltransferase (HAT) complex SAGA/ADA in *A. nidulans* (Nützmann *et al.*, 2011). We thus thought it possible that either MvIA or VeA might be involved in cross-talk with these complexes and/or chromatin remodelling of the *ors* cluster.

We assessed gene expression of two members of the SAGA/ADA complex, *gcnE* and *adaB* (Reyes-Dominguez *et al.*, 2008) that were found to be important for *S. rapamycinicus* activation of the *ors* cluster (Nützmann *et al.*, 2011). Both genes, *gcnE* more so than *adaB*, were modestly upregulated in the  $\Delta veA$  background but only *gcnE* subsequently dampened in the OE::mvIA $\Delta veA$  strain

(Fig. 5A). ChIP analysis of these strains reflected differential *gcnE* expression and activity as demonstrated by altered acetylation of the target residue H3K9 on two genes in the *ors* cluster (Fig. 5B). Analysis of the *orsD* promoter showed that the  $\Delta veA$  mutant displayed significantly increased levels of the activating H3K9ac mark. This increase was abolished in the OE::mvIA $\Delta veA$  strain. Similarly, acetylation of the *orsA* promoter in the OE::mvIA $\Delta veA$  is depressed relative to  $\Delta veA$ . This is in contrast to acetylation levels of the control *tubA* promoter, where there is no significant difference in acetylation among strains.

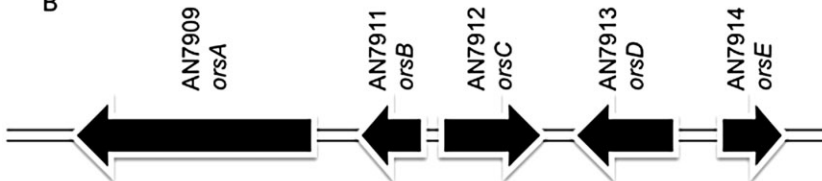
These data suggested that GcnE activity was important for *ors* cluster expression and F7995 production in the  $\Delta veA$  background. To more concretely assess this possibility, we next examined two  $\Delta veA\Delta gcnE$  double mutants. Both *ors* expression and F7995 production were lost in

A



**Fig. 4.** Gene expression analysis of *mvIA*, *veA*, and *ors* cluster genes of *A. nidulans* WT,  $\Delta veA$ , *OE::mvIA*, *OE::mvIA*  $\Delta veA$ , and  $\Delta mvIA$  (RDIT9.32, RJW112.2, RJW220.17, RJW221.5, and REC1C respectively) strains. 36, 60 and 48 represent hours of growth at 37°C, 250 r.p.m. after inoculation to GMM liquid media. Ethidium bromide-stained rRNA and *gpdA* are indicated for loading. The *ors* gene cluster is depicted at the bottom of the figure.

B



these strains (Fig. 6). Notably, the dark coloration of mycelium of the  $\Delta veA$  strain was remediated in the double mutants (Fig. S5B).

Since both H3K9 and K4 levels are impacted in the  $\Delta cclA$  background, we also asked if *mvIA* overexpression could limit F9775 production in the  $\Delta cclA$  background. As expected, and shown previously (Bok *et al.*, 2009), *cclA* loss resulted in an increase in F9775 production compared to wild type (Fig. 3D). Introduction of the overexpression *mvIA* allele into the  $\Delta cclA$  background (*OE::mvIA*  $\Delta cclA$ ) resulted in a reduction of F9775 production as compared to  $\Delta cclA$  alone. The fold reduction was similar to that of *OE::mvIA* in the  $\Delta veA$  background.

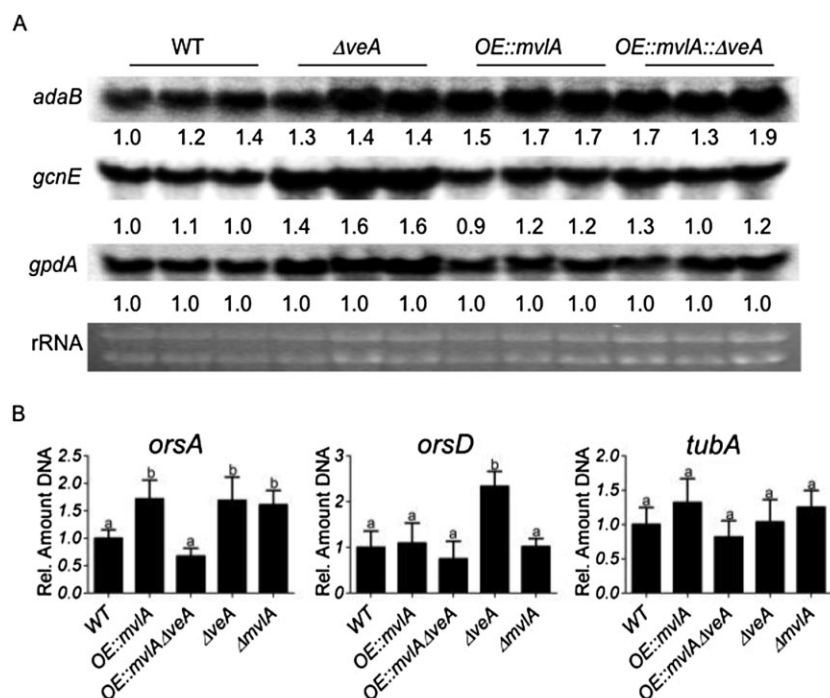
#### *veA* removal does not allow for increased F9775 production by two cluster activators

We were then interested to determine if *veA* loss – e.g. removal of a negative regulator of the *ors* cluster – would allow for further activation of the *ors* cluster by positive

secondary metabolite regulators. We chose two regulators which act through different mechanisms. RsmA is a bZIP transcription factor that increases sterigmatocystin and asperthecin production through promoter binding and activation (Yin *et al.*, 2012) whereas EsaA, a histone 4 acetyltransferase, has a more general mechanism in activating multiple secondary metabolite clusters through increased H4K12 acetylation (Soukup *et al.*, 2012a). Unexpectedly, examination of *OE::rsmA*  $\Delta veA$  and *OE::esaA*  $\Delta veA$  strains compared to their respective controls showed that both EsaA and RsmA overexpression resulted in an approximately twofold decrease in F9775 production in the  $\Delta veA$  background compared to  $\Delta veA$  alone (Fig. 3D).

#### *mvIA* and *ssmA* are not required for uracil catabolism

As noted earlier, both MvIA and SsmA shared significant identity to Urc4, a protein required for uracil utilization in *S. kluyveri*. Following yeast procedures (Andersen *et al.*, 2008), the single and double *mvIA* and *ssmA* deletion



**Fig. 5.** Transcriptional regulation of *gcnE*. *veA* deletion strain is decreased by overexpressed *mvIA*.

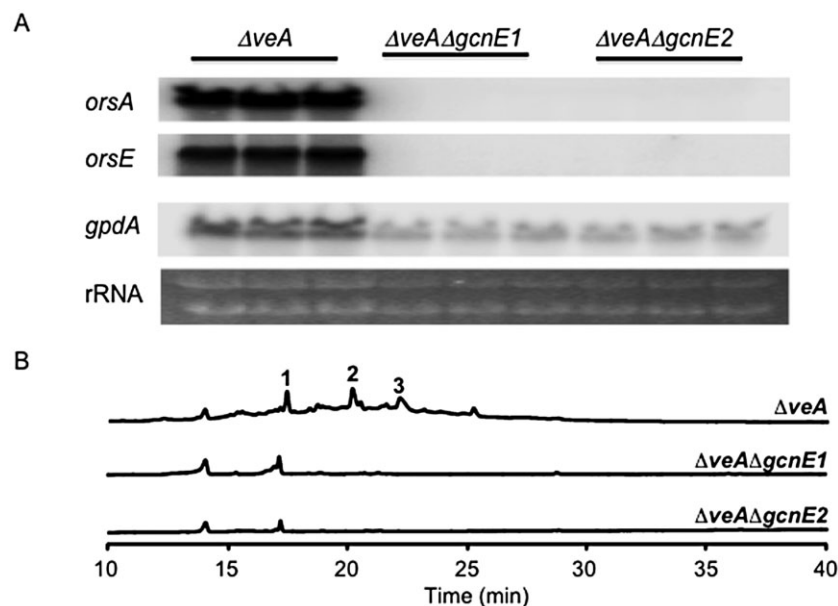
A. *adaB* and *gcnE* expression of *A. nidulans* WT,  $\Delta veA$ , *OE::mvIA* and *OE::mvIAΔveA* strains (RDIT9.32, RJW112.2, RJW220.17, and RJW221.5 respectively) strains grown on liquid GMM for 48 h at 37°C. RNA was extracted from independent biological triplicated samples. Ethidium bromide-stained rRNA and *gpdA* are indicated for loading. B. ChIP analysis of H3K9 acetylation levels at the *orsA*, *orsD*, and *tubA* promoters for 48 h cultures. Acetylation data has been normalized to total H3 in all cases. Letters represent significant differences at  $P < 0.05$ . Numbers below each lane are *gpdA*-normalized relative expression levels of the mutant strains in relation to the *gpdA*-normalized wild-type control, which has been arbitrarily set to 1.

mutants were examined for their ability to grow on uracil as a sole nitrogen source. Loss of *mvIA* was associated with decreased sporulation but this phenotype was independent of uracil supplementation (Fig. S7).

## Discussion

The nature of fungal secondary metabolite gene cluster regulation has been the topic of many recent investigations (Strauss and Reyes-Dominguez, 2011; Yin and Keller,

2011). Central to our understanding of cluster regulation has been the characterization of the conserved Velvet Complex whose members, particularly *laeA* and *veA*, are required for activation of dozens of secondary metabolite gene clusters (Bayram *et al.*, 2008; Amaike and Keller, 2009; Hoff *et al.*, 2010; Wiemann *et al.*, 2010; López-Berges *et al.*, 2012; Wu *et al.*, 2012; Yang *et al.*, 2013). While generally considered to be positive regulators of such clusters, a few studies have implicated these proteins in negative regulation of certain secondary



**Fig. 6.** Deletion of *gcnE* suppresses *ors* gene cluster expression and subsequent metabolite production in  $\Delta veA$ .

A. *orsA* and *orsE* expression in *A. nidulans*  $\Delta veA$  and  $\Delta veA\Delta gcnE$  (RJW112.2, TJW142.1, and TJW142.2 respectively) strains grown in liquid GMM for 48 h at 37°C. RNA was extracted from independent biological triplicated samples. Ethidium bromide-stained rRNA and *gpdA* are indicated for loading. B. Total scan HPLC profile of secondary metabolites extracted from the culture medium of *A. nidulans*  $\Delta veA$  and  $\Delta veA\Delta gcnE$  strains. Compound 1, 2 and 3 are the same structures shown as Fig. 3.

metabolites, for example a possible negative impact of VeA on penicillin production in *A. nidulans* (Kato *et al.*, 2003; Spröte and Brakhage, 2007) and Vel1 (the *Fusarium fujikuroi* VeA orthologue) on bikaverin production (Wiemann *et al.*, 2010). Here we describe our finding of a novel gene, *mvIA*, that together with *veA* negatively regulates the cryptic *ors* gene cluster.

The dark pigmentation of  $\Delta veA$  allowed for identification of *mvIA* in the multicopy suppressor mutagenesis screen (Fig. 2 and Fig. S1). We found that increased *ors* metabolite production contributed to this dark coloration. Both *OE::mvIA* and deletion of the polyketide synthase *OrsA* in the  $\Delta veA$  background yielded a decrease in pigmentation (Figs 2 and S5). The remaining pigmentation may be attributable to other mycelial pigments not regulated by VeA such as Met13 associated pigments (Fig. S6 and Frandsen *et al.*, 2010).

*MvIA* and *VeA* act at the transcriptional level to negatively regulate *ors* gene cluster expression. This was demonstrated by *ors* gene overexpression in  $\Delta veA$  and a delay in the overexpression when *OE::mvIA* was introduced into the  $\Delta veA$  background (Fig. 4). This regulation was reflected in product synthesis (Fig. 3). As examined here, this repressive impact of both proteins on the *ors* clusters appears to be at the level of chromatin modification, notably of histone 3 (Fig. 5). H3K9 methylation is a common heterochromatin mark (Strauss and Reyes-Dominguez, 2011) and decreased H3K9-2/3 methylation was also important in the induction of the *ors* cluster by *S. rapamycinicus* (Nützmann *et al.*, 2011). Specifically acetylation of this residue – the opposing euchromatin mark to methylation – by the SAGA/ADA complex was critical for activation of the *ors* cluster. This complex is not fully delineated in *A. nidulans* but at least two members, GcnE and AdaB, have been identified (Reyes-Dominguez *et al.*, 2008). A requirement for GcnE for an increase in *ors* cluster activation and subsequent F9775 production was supported by loss of both transcript and metabolites in the double  $\Delta gcnE \Delta veA$  strain.

A regulatory link between *veA* and the SAGA/ADA complex may occur in part at the transcriptional level. *veA* loss was associated with a modest increased transcript level of both *gcnE* and *adaB*; however only *gcnE* appeared dampened by *mvIA* overexpression in the  $\Delta veA$  background. Transcript levels of *ors* genes are increased in the  $\Delta veA$  strain, but absent in the  $\Delta gcnE \Delta veA$  double mutant, further suggesting that regulation of SAGA complex activity may be contributing to differential *ors* induction. To confirm that these alterations in transcript level reflected post-translational activity of the complex, we used H3K9ac as a marker for SAGA/ADA complex activity. H3K9ac was strongly enriched at the promoters of *orsA* and *orsD* in the  $\Delta veA$  strain, but decreased to wild type levels in the double mutant, mirroring what was seen at the transcript and

metabolite levels. These data confirm that acetylation of the *ors* cluster by the SAGA complex is necessary for cluster activation.

Interestingly, increased acetylation of *orsA* was also seen in both the *OE::mvIA* and  $\Delta veA$  strains, although transcript levels were unaltered in this strain. Therefore, H3K9 acetylation does not appear to be sufficient to activate transcription of this gene on its own. This confirms previous works of study on the orsellinic acid and sterigmatocystin clusters, where changes in histone modifications have been described that either failed to activate gene transcription (Nützmann *et al.*, 2011; Soukup *et al.*, 2012a), or dramatic changes in transcript levels did not correspond with altered modification of histone residues (Reyes-Dominguez *et al.*, 2010). Notably, the majority of these studies found modification of additional residues that correlated strongly with transcription, affirming the complexity of regulation, particularly regarding histone cross-talk.

The *ors* cluster was also shown to be activated by loss of CclA, a member of the H3K4 methylation Compass Complex (Bok *et al.*, 2009). Here the  $\Delta cclA$  mutant not only resulted in decreased H3K4-2/3 methylation – typically thought of as an activating mark – but also decreased H3K9-2/3 methylation. We also propose that decreased F9775 in the *OE::mvIA*  $\Delta cclA$  strain as compared to  $\Delta cclA$  alone reflects possible cross-talk in H3K4 and H3K9 modification activity.

Removal of the negative *ors* cluster regulator VeA did not allow for increased F9775 synthesis by two positive-acting secondary metabolite cluster regulators. Like GcnE and CclA, EsaA also is involved in chromatin modification, specifically acetylation of histone 4 residues (Soukup *et al.*, 2012a). A previous study showed that whereas *esaA* overexpression increased cluster gene transcription and product formation of the sterigmatocystin, terraquinone and penicillin gene clusters, there was no impact on the *ors* cluster nor its products. Our prediction that *OE::esaA* would increase F9775 production when *veA* was deleted was not borne out, at least not in the conditions utilized here. This may suggest that histone 4 acetylation does not impact *ors* cluster expression. Nor did overexpression of RsmA, known to increase production of sterigmatocystin and asperthecin, increase F9775 production in the  $\Delta veA$  background. This was not unexpected as RsmA has been found to increase sterigmatocystin, and hypothetically asperthecin, through activation of the sterigmatocystin specific transcription factor AfIR (Yin *et al.*, 2012). However, the decrease in F9775 production for both double mutants was not predicted. One explanation for this reduction in F9775 could involve decreased availability of metabolite precursors to *ors* cluster products as several of the RsmA and EsaA regulated metabolites draw on the acyl CoA pool. Indeed, studies have shown that inactivation of



one secondary metabolite can result in increased production of other secondary metabolites in both fungi and plants (Bok *et al.*, 2009; Post *et al.*, 2012; Wiemann *et al.*, 2012).

Beyond its apparent repressive affect on GcnE activity and H3K9 acetylation levels in the  $\Delta veA$  background, the mechanism of MvIA remains enigmatic. Despite its fairly high identity to Urc4, a protein involved in uracil metabolism in *L. kluyveri* (Andersen *et al.*, 2008; Björnberg *et al.*, 2010), our study did not indicate a role for MvIA, or SsmA, in pyrimidine metabolism in *A. nidulans*. MvIA is conserved in fungi and it will be interesting to see if there may be a role for this protein in VeA interactions in other fungi.

## Experimental procedures

### Strains and growth conditions

Strains used or created in this study are listed in Table S1. All strains unless otherwise noted were grown on glucose minimal media, GMM (Shimizu and Keller, 2001), with additional supplements for auxotrophic strains (pyrodoxin, riboflavin, uridine, uracil, or para aminobenzoic acid as needed). The genetic background of the primary strains used in this study are as follows: RDIT9.32 (WT) and RJW112.2 ( $\Delta veA$ ) are isogenic prototrophs used for expression and physiology studies. RJW113.4 ( $\Delta veA$ ) is an auxotroph (*pyrG89*) used for sexual crossing and mutagenesis. RJW112.2 and RJW113.4 were obtained from sexual cross between RDIT47.32 ( $\Delta veA$ , *trpC801*) and RTMH207.13 (*pyrG89*, *veA+*). RDIT47.32 is a sexual cross progeny of DVARI X RTMK30.23. RDIT30.23 is from FGSC237 X RDIT1.1. RDIT1.1 is from pW1 X FGSC773. All strains are maintained as glycerol stocks at  $-80^{\circ}\text{C}$ .

### Transformation with AMA1 genomic DNA library

The recipient strain, RJW113.4, was transformed with the pRG3-AMA1-based *A. nidulans* wild type genomic DNA plasmid library with *pyr4* of *N. crassa* as the selective marker (Shaaban *et al.*, 2010). Transformation techniques followed previously described procedures (Shaaban *et al.*, 2010). Thousands of transformants were screened for pigment reduction on the underside of the fungal colonies. Total DNA from individual transformants was used to amplify insert by polymerase chain reaction (PCR) with primers flanking the DNA insert in the AMA1 plasmid (AMAFWD and AMAREV, Table S2). The PCR amplicons were sequenced, and the assembled sequences were used for BLASTn search of the *A. nidulans* genome (<http://www.broadinstitute.org>). Plasmid pJW100 was rescued from one of the transformants and re-transformed into strain RJW113.4 to confirm reduction of pigmentation. The sequence of the insert in pJW100 was confirmed one more time by BLAST searching of the *Aspergillus* comparative database.

### Construction of *mvIA* deletion, overexpression and double mutants

The *mvIA* deletion strain (TJW98) was created in RJMP1.49 by replacing AN8797 (*mvIA*) ORF with *A. parasiticus pyrG*

using modified double joint PCR (Yu *et al.*, 2004) consisting of the following: 1 kb DNA fragment upstream of the *mvIA* start codon (primers 8797F5 and 8797PR5), a 2 kb DNA fragment of *A. parasiticus pyrG* (primers parapyrGF and parapyrGR) via pJW24 (Calvo *et al.*, 2004), and a 1 kb DNA fragment downstream of the *mvIA* stop codon (primers 8797PF3 and 8797R3). First round PCR protocol started with an initial activation of the Pfu Ultra fusion II polymerase (Agilent) at  $95^{\circ}\text{C}$  for 3 min with hot start. For the next 36 PCR cycles, the temperature was held at  $95^{\circ}\text{C}$  for 30 s denaturing, then ramped up to  $58^{\circ}\text{C}$  for 30 s annealing, and to  $68^{\circ}\text{C}$  for 2 min extension. Finally, a post-extension step was performed at  $68^{\circ}\text{C}$  for 10 min. Second round PCR utilized gel-purified DNA templates from first round PCR with Pfu Ultra fusion II polymerase at  $95^{\circ}\text{C}$  for 3 min with hot start. For the next 11 PCR cycles, the temperature was held at  $95^{\circ}\text{C}$  for 40 s denaturing, then ramped to  $60^{\circ}\text{C}$  for 3 min annealing, and to  $68^{\circ}\text{C}$  for 4 min extension. Finally, a post-extension step was performed at  $68^{\circ}\text{C}$  for 10 min. Third round PCR started with Expand long template PCR system (Roche) at  $94^{\circ}\text{C}$  for 3 min. For the next 36 PCR cycles, the temperature was held at  $94^{\circ}\text{C}$  for 20 s denaturing, then ramped to  $59^{\circ}\text{C}$  for 30 s annealing, and to  $68^{\circ}\text{C}$  for 4 min extension. Finally, a post-extension step was performed at  $68^{\circ}\text{C}$  for 10 min. 30  $\mu\text{l}$  of G50 purified third round PCR product was used for fungal transformation. *mvIA* deletants were confirmed by PCR and Southern blot and one correct transformant, TJW98, used for sexual crosses. TJW98 was sexually crossed with RTMH207.13 to obtain the prototroph REC1C. The recombinants were confirmed with PCR.

A *mvIA* overexpression (OE) cassette, pJW111.58, was created by inserting a single joint PCR product (1.4 kb DNA fragment of *A. nidulans gpdA* promoter (primers 8797GPDF and 8797PR5), a 2.4 kb DNA fragment containing the *mvIA* ORF and termination cassette (primers 8797OEF and 8797OER) into the *HindIII* and *SpeI* sites of pJW53 which resulted in the fusion of the *mvIA* gene with the *gpdA* promoter. pJW111.58 was used to transform RJMP1.31 to create an overexpression *mvIA* allele in the *pyrA* locus. The resulting strain was called TJW99. We confirmed TJW99 by PCR and Southern blotting and then sexually crossed TJW99 with RJW113.4 to create a prototrophic strain (REC2C). Subsequent northern analysis was done using radiolabelled probes for the corresponding transcript (1 kb DNA fragment, primers 8797IF and 8797IR).

The  $\Delta veA::mvIA$  double mutant strain was created by crossing TJW99 with RJW113.4 to obtain RJW221.5 and confirmed with selective media and PCR. All of primers used in this study are listed in Table S2.  $\Delta laeAOE::mvIA$  and  $\Delta cclAOE::mvIA$  double mutants were created by crossing TJW99 with RJW135.1 and RJW122.2 to obtain RJW223.2 and RJW254.12 respectively. Strain RAAS44.15 was obtained through mating of TAAS16.13 (Soukup *et al.*, 2012a) with DVARI (Kim *et al.*, 2002).

### Construction of *ssmA* deletion, overexpression, and double mutants

AN12027 (*ssmA*) was deleted by replacing it with *A. fumigatus riboB* in *A. nidulans* strain TNO2A7 using modified double joint PCR. Construction of *ssmA* deletion, overexpression and double mutants consisted of the following: 1 kb DNA



fragment upstream of the *ssmA* start codon (primers 12027F5 and 12027R5), a 2.3 kb DNA fragment of *A. fumigatus riboB* (primers 12027rb5' and 12027rb3'), and a 1 kb DNA fragment downstream of the *ssmA* stop codon (primers 12027F3 and 12027R3). The deletion mutants were confirmed by PCR and Southern blot and one deletant, TJW124.13, was sexually crossed with RDIT2.1 to get a prototrophic strain (RJW215.25) and a *metG* auxotrophic strain (RJW215.9). RJW215.9 was sexually crossed with TJW98 to obtain a  $\Delta mvlA\Delta ssmA$  double deletion mutant (RJW229.2). A *ssmA* overexpression mutant was created with *A. parasiticus pyrG* with double joint PCR product consisting of the following: 1 kb DNA fragment upstream of the *ssmA* start codon (primers 12027F5 and 12027oe5'R), a 3.6 kb DNA fragment of *A. parasiticus pyrG* (primers parapyrGF and parapyrGR) with *A. nidulans gpdA* promoter via pJMP9 (Soukup *et al.*, 2012b) and a 1 kb DNA fragment downstream of the *ssmA* start codon (primers 12027oe3'F and 120273'R). The transformants were screened by PCR and Southern blot to obtain TJW125.1. This mutant was sexually crossed with RDIT2.1 and RJW113.4 to get a overexpression prototroph RJW216.15 and a *OE::ssmA* $\Delta veA$  double mutant (RJW226.4) respectively. Subsequent northern analysis was done using radiolabelled probes for the corresponding transcript (1 kb DNA fragment, primers 12027IF and 12027IR). All primers used in this study are listed in Table S2.

#### Construction of *veA* and *gcnE* double deletion mutants

The double deletion strain (TJW142) was created in RJW113.4 by replacing AN3621 (*gcnE*) ORF with *A. parasiticus pyrG* using modified double joint PCR consisting of the following: 1 kb DNA fragment upstream of the *gcnE* start codon (primers gcnE5F and gcnE5R), a 2 kb DNA fragment of *A. parasiticus pyrG* (primers parapyrGF and parapyrGR) via pJW24 (Calvo *et al.*, 2004), and a 1 kb DNA fragment downstream of the *gcnE* stop codon (primers gcnE3F and gcnE3R). 30  $\mu$ l of G50 purified third round PCR product was used for fungal transformation. Double deletants were confirmed by PCR and two correct transformants, TJW142.1 and 142.2 used for subsequent experiments.

#### Construction of green fluorescent tagged *mvlA*

A N-terminus GFP tagged *mvlA* cassette was created through two steps. First, a DNA fragment of the *mvlA* ORF (1.7kb, primers 8797gfpGpdF and 8797gfpGpdR) was inserted in between the *gpdA* promoter and *gfp* of pJMP50 (*A. fumigatus pyroA* with *A. nidulans gpdA* promoter and a fused *gfp*) to make pJW147.17 using Quick change method (Bok and Keller, 2012). For creation of pJMP50, the *ptrA* gene was removed from pSK505 by restriction digestion with *SphI* and *PstI*. Subsequently, the *A. fumigatus pyroA* gene was amplified using the oligonucleotides JP Af pyroA *SphI* For (5' ACATCAGcATGCTGGATTACTAAGGTAATG) and JP Af pyroA *PstI* Rev (5' AGTActGCAGCATCCACATGATC) and cloned into pCR TOPO Blunt II (Invitrogen) to construct pJMP61. A 1.8 kb *SphI-PstI* fragment corresponding to the *A. fumigatus pyroA* gene was cut out of pJMP61 and ligated

into the *SphI-PstI* sites of pSK505 to construct pJMP50. The *gpdA* promoter of pJW147.17 was then replaced with a *mvlA* promoter (1kb, primers 8797gfpnatF and 8797gfpnatR) by Quick change method to create the plasmid pJW149.7. This plasmid was confirmed by sequence analysis to contain a *mvlA* promoter, N-terminus *gfp* and *mvlA* ORF, and then used to transform TJW98 to create the strain TJW106. Dark cultured germlings were viewed at 40 $\times$  magnification using a fluorescence microscope (Zeiss AxioImager.A1) and AxioVision image capturing software. Germlings were grown for over night at 30°C in liquid GMM over a coverslip. The coverslip was turned over onto a slide and viewed immediately. Hoechst dye was used to identify nuclei for the N-terminus GFP tagged *MvlA* strain TJW106.

#### Nucleic acid analysis

DNA extraction, restriction enzyme digestion, gel electrophoresis, blotting, hybridization, and probe preparation were performed by standard methods (Sambrook and Russell, 2001). RNA extractions were made from mycelia of cultures where  $10^5$  spores ml<sup>-1</sup> were grown in 50 ml liquid GMM at 37°C with shaking at 250 r.p.m. for 48 h. For pigment and pigment associated gene (*yA*, Aramayo and Timberlake, 1990; *cfwA*, Marquez-Fernandez *et al.*, 2007; *met13*, Frandsen *et al.*, 2010) expression studies,  $1 \times 10^6$  spores ml<sup>-1</sup> of strains was inoculated into 50 ml of liquid GMM and grown for 24 h at 37°C and 250 r.p.m. shaking. Mycelia were harvested by filtration through Miracloth (Calbiochem) and transferred onto solid GMM for additional 6 h and 20 h. Total RNA was extracted using Isol-RNA Lysis Reagent (5 Prime) according to the manufacturer's instructions with approximately 30  $\mu$ g of total RNA for RNA blot analysis. RNA blots were hybridized with *mvlA*, *ssmA*, *veA*, *orsA*, *orsB*, *orsC*, *orsD*, *orsE*, *gcnE*, *adaB*, *yA*, *cfwA*, *met13* and *gpdA* DNA fragments which were generated by PCR using gene-specific primers as shown in Table S2. Expressed gene intensity was quantified using ImageJ software.

#### Metabolite assays

Thin layer chromatography (TLC) was used to assess sterigmatocystin (ST) production. ST was extracted from GMM solid medium culture, which was point-inoculated with  $10^3$  spores per plate and grown for 5 days at 37°C. One cm cores were punched from the centre of point inoculated plates and homogenized with 3 ml ddH<sub>2</sub>O. Three millilitres of chloroform was added and samples were centrifuged for 10 min. The organic layer was removed and put into a 3 ml glass vial and left to sit and dry in a fume hood overnight. Dried extracts were resuspended with 100  $\mu$ l chloroform and 5 or 10  $\mu$ l were loaded onto a non-UV coated TLC plate. ST was spotted as a standard. The plates were run in chloroform : acetone (8:2) solvent and stained with 15 % aluminum chloride in 95 % ethanol. TLC plates were viewed under 254 nm UV light.

Penicillin bioassays were carried out with fungal culture filtrate (10 ml) harvested from  $10^5$  spores ml<sup>-1</sup> liquid-shaking culture after 120 h at 37°C, 225 r.p.m. Freeze-dried culture filtrate was resuspended in 1 ml ddH<sub>2</sub>O and 200  $\mu$ l of resuspended sample was added into 1 cm diameter wells in a

TSA + *Micrococcus luteus* bacteria plate (15 g Bacto Tryptone, 5 g Bacto Soytone, 5 g NaCl, 10 g agar per litre of media, with 500 µl *M. luteus* (OD = 1) added to 40 ml TSA per plate). Plates were left at 4°C for 3 h then incubated at 37°C overnight. Where indicated, 12 U of penicillinase was added to 200 µl of extracts and incubated for 3 h at 37°C before adding to wells as a control.

#### Fermentation and LC/MS analysis

*Aspergillus nidulans* wild type and mutant strains were cultivated at 37°C, 225 r.p.m. in 50 ml of liquid GMM at  $10^5$  spores ml<sup>-1</sup>. After 48 h or 5 days, culture broth was harvested by filtration and 40 ml lyophilized. The lyophilized sample was suspended in H<sub>2</sub>O (20 ml) and partitioned with ethyl acetate (20 ml). In order to extract acidic phenolic metabolites, the water layer was acidified to pH 2 and partitioned with ethyl acetate (20 ml) a second time. The combined ethyl acetate layers were evaporated in vacuo, re-dissolved in 0.5 ml of dimethylsulphoxide/methanol (1:4) and injected with 10 µl for HPLC-DAD-MS analysis. LC/MS was carried out in negative mode using a ThermoFinnigan LCQ Advantage ion trap mass spectrometer with an RP C18 column (Alltech Prevail C18 3 µm 2.1 × 100 mm) at a flow rate of 125 µl min<sup>-1</sup> as described (Bok et al., 2009).

For F9775A and B quantification, linear curves were generated by using UV-Vis 430 nm as detection. The extracts were prepared so that the concentration of F9775A and B lay within the standard curves.

#### Chromatin immunoprecipitation and real time qPCR analysis

Fifty millilitres cultures of liquid GMM was inoculated with  $1 \times 10^6$  spores per ml and incubated at 250 r.p.m. and 37°C for 24 h under light. Triplicate cultures were performed for each strain. Chromatin immunoprecipitation was carried out as described previously (Boedi et al., 2012). Antibodies used for ChIP were: rabbit polyclonal to histone H3 acetyl K9, Abcam, ab10812 and rabbit polyclonal to C-terminus histone H3 antibody, Abcam, ab1791. Two µg of antibody was used per reaction of 200 mg total protein. Amplification and detection of precipitated DNA in real-time qPCR was performed with iQ™ SYBR® Green Supermix (Bio-Rad, Cat#170-8880) following the manufacturer's instructions using primers listed in Table S2. Relative amounts of DNA were calculated by dividing the immunoprecipitated DNA by the input DNA. Each PCR reaction was replicated. To normalize the amount of DNA precipitated with histone H3-acetyl K9 the quantities from precipitation with this antibody was divided by the previously calculated ratio of the anti-C-terminus histone H3 precipitation to input DNA. All values are shown relative to wild type. To ensure that levels of precipitated DNA were above background, Rabbit IgG Abcam, ab27478 was used as a non-specific control. In all cases, precipitated DNA levels were at least 100-fold above background (data not shown).

#### Pyrimidine utilization assay

Uracil or uridine (2 g l<sup>-1</sup>) was supplied as a nitrogen source in the solid medium containing Difco yeast nitrogen base without

amino acid or Difco yeast nitrogen base without amino acid and ammonium sulphate 2.5 g l<sup>-1</sup>, glucose 10 g l<sup>-1</sup>, *A. nidulans* trace element 1 ml l<sup>-1</sup> (1000-fold stock) and 1.5 % agar as modified ingredient published by Andersen et al. (2008). One microlitre of  $10^6$  conidia ml<sup>-1</sup> was inoculated on the centre of media and strains cultured 10 days in a 37°C incubator.

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